Development of novel small-size peptides as putative therapeutic drugs

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Chapter 8

C8

General discussion & future perspectives

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8.1 The role of computational medicinal chemistry in today's drug discovery

It is well known that the ultimate goal of the medicinal chemist is to develop, by rational design, a molecule which will produce a desired biological activity without producing undesirable collateral effects. To be able to achieve such an objective a complete description of the pharmacophore (box 8.1) is extremely important. Although the presently available techniques of molecular modeling have allowed more "rational" and planned investigation with respect to those developed in the last century, they are still far from perfect. However, computational chemical techniques are used routinely to identify underlying reasons and explanations for observed chemical reactivity, or to assess the probability of a molecule to exhibit certain desired properties. Thus, *in silico* (box 8.2) techniques reduce the human effort, the time consumed in bioassays, and the number of experimental animal laboratory studies necessary to provide the first steps towards developing a new drug. In order to achieve the above mentioned goals, compromises in view of the complexity of the biological system and individual variations must be taken into account. Otherwise, a realistic approach of a given system may be put at risk.

Box 8.1: Pharmacophore definitions
- P. Ehrlich, (1909); "a molecular framework that carries (phoros) the essential features responsible for a drug's (pharmacon) biological activity".
- P. Gund, (1977); "a set of structural features in a molecule that is recognized at a receptor site and is responsible for that molecule's biological activity"
- IUPAC (1998); "an ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target and to trigger or block its biological response".

Besides being a very interesting source of new potential drugs, peptides are still relatively large sized systems which seriously limit the complexity of the level of theory that is going to be used to attack the problem from the point of view of the computational technique. Normally, a hybrid methodology that combines molecular dynamics with quantum mechanical geometry optimization is nowadays used. Complex molecular systems can be approached by using this methodology in a relatively realistic fashion. Also, a reduced model may be generated from the former one in order to perform more exact energetic determinations by using quantum calculations. Thus, the "whole" system can be studied by splitting it into several reduced models. It is for this reason that this reductionist approach is generally called a "divide and conquer" challenge.

On the other hand, the rapidly evolving and improving cost-performance relationship is placing high-tech computers on the desktop of today's medicinal chemist. Access to high resolution interactive computer graphics, conformational energy minimization of relatively larger biological systems, classical and quantum molecular dynamics, geometrical optimizations, homologous protein structure generation and sequence database searching are examples of some tools which can routinely accessed through commercial molecular modeling software packages. All in all, these improvements allow us to perform more realistic simulations of the biological system under study.

The application of molecular modeling techniques in any area of medicinal chemistry have been shown to be convenient and necessary in order to determine the potential compounds to be synthesized and tested. Some great findings have been accomplished regarding drug design based on molecular modeling. For instance, Agouron Pharmaceutical designed Viracept, an HIV-1 protease inhibitor, using structure-based design tools (Babine et al. 1995). Crixivan (Dorsey et al. 1994), another HIV-1 protease inhibitor received FDA approval in record time, and is now used by over 300,000 patients worldwide. Also, by means of structure-based methods, Merck developed a glaucoma therapeutic agent called Trusopt (Greer et al. 1994). Without such calculations, neither structure based approaches nor *de novo* drug design would be at the stage that they are at present. In addition, these techniques helped to design several new candidate drugs as mentioned above.

I personally consider from a scientific point of view that the use of molecular modeling supporting experimental bioassays is not only preferable, but also necessary. It has been recognized that scientific knowledge has advanced far enough to permit a focus on molecular mechanisms with atomistic details. In today's drug discovery *in silico* molecular modeling studies are now developed and fully accepted, not as a supplement to a checklist procedure, but rather as integral part of medicinal chemistry.

Box 8.2: *In silico* is an expression used to mean "performed on computer or via computer simulation." The phrase is coined in analogy to the latin phrases *in vivo* and *in vitro* which are commonly used in biology and refer to experiments done in living organisms and outside of living organisms, respectively. The term *in silico* does not mean anything in latin. The proper latin phrase would likely be in simulacro to describe experiments done on the likeness (simulacrum) or model of a phenomenon.

8.2 cAMP signaling pathways as a putative new target of antifungal drugs

Amongst the fungi tested as part of the present thesis, *Cryptococcus neoformans* (box 8.3) and *Candida albicans* (box 8.4) were the most sensitive to the antifungal peptides designed in this thesis. It is important to highlight that the mechanism of action of these peptides has not been determined yet. Nevertheless, as a general feature, antimicrobial cationic peptides possess a relatively non-specific mechanism of action by either acting through a detergent-like disruption of the bacterial or fungal cell membrane or by the formation of transient transmembrane pores (Huang 2000; Shai 2002). The former mechanisms are the ones proposed especially for cell-penetrating peptides studied in chapter 4, since these peptides are long enough to form α-helix conformations that may lead to cell membrane pore formation. However, peptides derived from the α-MSH studied in chapters 2-3 may be too short to form cell membrane pores. Nevertheless, there is now evidence that the antimicrobial activity of α-MSH and derivative peptides is exerted through a unique mechanism substantially different from that of other natural antimicrobial peptides, at least in the case of *C. albicans* (Grieco et al. 2003).

Previous investigations have yielded evidence that the candidacidal effect of α-MSH is linked to mechanism of cAMP-inducing activity. α-MSH seems to increase cAMP production in *C. albicans* and the adenylyl cyclase inhibitor ddAdo (dideoxyadenosine) partly reversed the candidacidal effect of the peptide (Cutuli et al. 2000; Grieco et al. 2003). It is remarkable, however when we carefully look at it, this mechanism of action in *C. albicans* mimics the influence of α-MSH in mammalian cells in which the peptide binds to G-protein-linked melanocortin receptors, activates adenylyl cyclase, and increases cAMP. In support of this, Singh and coworkers reported that, by using phosphodiesterase inhibitors, the alteration in the cAMP signaling pathway affects the cell cycle progression in *Candida albicans* (Singh et al. 2007). Therefore, implication of cAMP signaling in both the cell cycle and morphogenesis seems to be a potential target for the development of new antifungal drugs. However, due to the overall implications that cAMP signaling pathways have in humans, one may ask; how selective the designed drugs using this target would be? Since, fungal cells seem to have similar cAMP signaling pathways as human cells, a compound that targets this pathway would not be, at least in principle, a selective enough candidate.

Box 8.3: *Cryptococcus neoformans* is an encapsulated yeast-like fungus that can live in both plants and animals. This species belongs to the broad class of organisms called "club fungi" or division basidiomycota, which is one the five major types of fungi. *C. neoformans* usually grows as a yeast (unicellular) and replicates by budding. Under certain conditions, both in nature and in the laboratory, *C. neoformans* can grow as a filamentous fungus. When grown as a yeast, *C. neoformans* has a prominent capsule composed mostly of polysaccharides. Infection with *C. neoformans* is termed cryptococcosis. Most infections with *C. neoformans* consist of a lung infection. However, fungal meningitis, especially as a secondary infection for AIDS patients, is often caused by *C. neoformans* making it a particularly dangerous fungus. Infections with this fungus are rare in those with fully functioning immune systems. For this reason, *C. neoformans* is sometimes referred to as an opportunistic fungus.

Box 8.4: *Candida albicans* is a yeast fungus and a causal agent of opportunistic oral and genital infections in humans. Systemic fungal infections, especially candidiasis, have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g., AIDS, cancer chemotherapy and organ or bone marrow transplantation). *C. albicans* is among the many organisms that live in the human mouth and gastrointestinal tract. Under normal circumstances, *C. albicans* lives in *ca* 80% of the human population with no harmful effects, although overgrowth results in candidiasis.

8.3 Targeting the amyloid hypothesis with Aβ-toxicity offsetting peptides

It is controversially discussed whether the amyloid fibrils are an epiphenomenon linked to Alzheimer's disease (AD) or whether fibril formation causes AD (Forman et al. 2007; Wisniewski et al. 1997). There is now more consensus, however, based on *in vitro* and *in vivo* evidence that soluble oligomeric forms of Aβ have potent neurotoxic activity and are the primary causes of neuronal injury and cell death occurring in AD (Lesne et al. 2006; Lue et al. 1999; Naslund et al. 2000; Wasling et al. 2009). On the other hand, there is a relatively weak correlation between the severity of dementia and the density of fibrillar amyloid plaques (Dickson et al. 1995; Katzman 1986; Terry et al. 1991) and indeed this has been frequently cited as a critical flaw in the Aβ hypothesis. However, several studies have shown a robust correlation between soluble Aβ levels and the extent of synaptic loss and severity of cognitive impairment (Chang et al. 2006; Chapman et al. 1999b; Dineley et al. 2002; Dodart et al. 2002; Haass and Selkoe 2007; Jacobsen et al. 2006; Lesne et al. 2006; Lue et al. 1999; McLean et al. 1999; Naslund et al. 2000; Shankar et al. 2008; Shankar et al. 2007b; Walsh and Selkoe 2007; Wang et al. 1999; Wasling et al. 2009).

It is important to mention that the production of Aβ is a normal process (Haass et al. 1992a; Seubert et al. 1992; Shoji et al. 1992), but in a small number of individuals, the overproduction of all Aβ species, or an increased proportion of the 42 amino acids form, appears sufficient to cause early onset AD (Bentahir et al. 2006; Cai et al. 1993; Citron et al. 1992; Haass et al. 1992b; Kumar-Singh et al. 2006; Roveletlecrux et al. 2006; Seubert et al. 1992; Suzuki et al. 1994). Therefore, the mere presence of Aβ does not cause neurodegeneration; rather neuronal injury appears to ensue as a result of the ordered self-association of Aβ molecules (Busciglio et al. 1992; Geula et al. 1998; Pike et al. 1991). However, it should be appreciated that besides its pathological properties, Aβ may have important roles in synaptic plasticity and normal brain functioning (Pearson and Peers 2006; Puzzo et al. 2008; Wasling et al. 2009). The endogenous level of Aβ in the brain is regulated by synaptic activity *in vivo*, suggesting a dynamic feedback loop involving APP metabolism and Aβ that may modulate synaptic activity (Haass and Selkoe 2007). Thus, it has been shown that the depletion of endogenous Aβ, by a single intrahippocampal administration of anti-Aβ-antibody, leads to disrupted memory retention in rats (Garcia-Osta and Alberini 2009).

The advantage of targeting the amyloid hypothesis with Aβ-toxicity offsetting peptides (BOX 8.5), in comparison to other putative therapeutic approaches for AD such as vaccination, is that they specifically target the abnormal conformation of Aβ without interfering with any possible physiological function of the soluble monomeric Aβ peptide. Moreover, this type of compounds may not interfere with the metabolism of APP, and this is an advantage in comparison with the secretase inhibitors (e.g.: β-secretase and g-secretase inhibitors). Blocking this secretase system can cause failures of very important metabolic pathways. Thus, blocking γ-secretase complex, besides lowering Aβ formation in experimental systems, interferes with receptor/signalling system of the protein Notch. The reduction of Notch activity could interfere with important cellular proliferation and differentiation pathways (Pollack and Lewis 2005).

Box 8.5: The term Aβ-toxicity offsetting peptides is used in this thesis to refer to those peptides that somehow, neutralize the toxic effect of Aβ on neurons. The term "*β-sheet breakers*", introduced by C. Soto in 1998, have been widely used to refer to this kind of peptides, even when the peptides did not show any β-sheet breaking properties. For this reason, I propose to use this new terminology to refer to this novel class of compounds, in which the effect of the peptide is mentioned but not necessarily its way of action.

On the other hand, it was shown that Leu-Pro-Tyr-Phe-Asp-NH_2 and PN22 peptides, in respectively CHAPTERS 6 and 7, may bind the monomeric form of $A\beta_{42}$. Nevertheless, this binding property may interfere with the conformational changes that precede the aggregation process and not with the normal physiological functions of the soluble monomeric Aβ peptide.

8.4 May anti-amyloid properties lead to antifungal activity?

Some of the Aβ-toxicity offsetting peptides studied here were designed on the basis of their capabilities to bind Aβ aggregates by "mimicking" Aβ properties and conformational behavior (CHAPTERS 6 and 7), which might endow them anti-amyloid properties. On the other hand, the antifungal peptides ported in CHAPTERS 2-4 share some structural similarities with these Aβ-offsetting peptides. For instance, both types of peptides are positively charged and have a compromising relationship between hydrophobic/aromatic residues and charged residues. However, they do not necessarily share the same conformational preferences. In general, cell-penetrating peptides with antifungal properties (CHAPTER 4) showed a preference to form folded conformations (a-helix-like conformations), while α-MSH derived antifungal peptides (CHAPTERS 2-3) preferred extended conformations (β-sheet-like conformations). The latter one is another similarity shared with the Aβ-toxicity offsetting peptides reported in CHAPTER 7. Regarding the above mentioned, one may wonder whether anti-amyloid properties can lead to antifungal activity?

Within the past two decades, several proteins that do not necessarily have similar amino-acid sequences have been reported to form amyloid-like aggregates on the surfaces of some fungi and bacteria (Claessen et al. 2003; Claessen et al. 2002; Gebbink et al. 2005). The functions that these aggregated proteins seem to play on the surfaces of these microorganisms are multifold. For instances, these amyloids play an important role in the invasion of abiotic and biotic substrates, reduce the water surface tension allowing hyphae to escape aqueous environments, and enable hyphae to penetrate solid surfaces (e.g.: host cuticle) amongst others. There is also experimental evidence that the amyloid layer forms a protective "coat" to allow microorganisms to evade the immune system of the host (Wosten and De Vocht 2000). Currently, hydrophobins are the only proteins known to form amyloids on fungal surfaces (Linder 2009). Other proteins may fulfill this role in fungi that do not express hydrophobins (e.g.: *Candida albicans*) (Gebbink et al. 2005). Bacteria have been shown to form cell-surface-located amyloids of two classes of proteins; chaplins and curli/tafi (Claessen et al. 2003; Gebbink et al. 2005). Thus, it seems that the presence of amyloid structures on the cell surface of microorganisms is widespread. Therefore, these amyloid-like arrangements seem to be a promising novel target to design compounds capable of disrupting the structure of these aggregates and through this action to expert their anti-microbial properties. In that respect, *C. neoformans* seems to be highly affected by the peptides reported in this thesis (CHAPTERS 2-4). May be this due to an existing amyloid "coat" on the surface of this fungus, which may be disrupted by these peptides? Whether this is the case of *C. neoformans* or not, the finding of such structures on the surface of pathogenic microorganisms that cause life-threatening infections, is a novel target to develop selective therapeutic agents.

8.5 Overall conclusions and perspectives

The work described in this thesis is a step forward towards a protein based drug design in which methods of rational drug design and dynamical features of the target have been taken into account. Clearly, the *in silico* design is a method that can be used to study complex biological systems. However, the results of the simulations should be interpreted with caution.

Several new targets to develop putative drugs have been mentioned in this thesis, which may give the possibility to project future investigation programs, some of them are already being in a developing phase. With regards to antifungal peptides, new families, based on our results reported in CHAPTER 4, are being designed, synthesized and tested. Regarding the Aβ-toxicity offsetting peptides, new structures have been proposed as well as peptidomimetic structures currently under investigation in our labs. We may conclude with the statement that the findings presented in this thesis hold great potential for further development of a strategy towards therapeutic applications in two major human health conditions. ▪

REFERENCES